EXHIBIT D
Application Serial No. 09/089,871

ENZYMES IN ANIMAL NUTRITION

Roche

Proceedings of the 1st Symposium
Kartause Ittingen, Switzerland
October 13 - 16, 1993

lacillus subtilis (natto)

:os, D., Wolters, M.,

EXPERIENCES WITH ENZYMES IN FEED MANUFACTURING

M. Gadient, L. Völker and W. Schüep
F. Hoffmann - La Roche Ltd
Vitamin Division
Research and Technology Development
Basel, Switzerland

Summary

Studies were conducted to assess the influence of hydrothermal processes used during practical feed manufacturing on the stability and efficacy of supplemented carbohydrases. Enzyme activities of feeds were determined analytically, and the performance of broiler chicks fed diets supplemented with various enzyme sources was monitored. The results obtained indicate that the stability of the tested carbohydrases is not endangered to a great extent, if a critical temperature of around 75 °C is not exceeded during the hydrothermal process. With higher process temperatures, stability and efficacy of enzymes are sizeably reduced. High temperature stress can be avoided by adding the enzymes after the hydrothermal process.
(Keywords: enzyme, assay, stability, feed, feed manufacturing)

Introduction

Enzymes belong to the micro-ingredients sensitive to feed manufacturing processes. Among these, hydrothermal processes, such as pelleting, extrusion and expansion, have been recognised as potentially destructive for carbohydrases, e.g. glucanases (Campbell and Classen, 1989), phytase (Jungbloed and Kemme, 1990; Schwarz and Schöner, 1991) and proteases (CRC Handbook, 1980). Controversy exists as to the extent of these losses, which appear to be affected by the type of enzyme preparation as well as the methods of assessing pelleting temperature and enzyme recovery.
Studies were carried out with commercial carbohydrases in order to quantify the losses and to identify ways to reduce them.

Materials and methods

Experimental products. The experiments were run with 4 commercial multi-enzyme preparations (Table 1): A) dry enzyme complex derived from *Trichoderma viride*, B) a dry enzyme of fungal and bacterial origin, C) a dry enzyme of fungal and bacterial origin, D) a liquid form derived from *Trichoderma longibrachiatum*.

Experimental diets and design. Three trials were conducted to assess:
(1) the physical and chemical characteristics of the dry enzymes
(2) the recovery and stability of the enzymes in feed, monitored by analytical and biological parameters

(3) the use and efficacy of an enzyme in liquid form.
Particle size and number were measured with a laser particle sizer. For assessing thermostability, the products were moistened to reach a water content of 15% and heated for 30 minutes at 95 °C. Enzyme activities were measured before and after the treatment.
For experiment 2, a commercial broiler feed based on barley, wheat and rye (composition and contents in Table 2) was mixed and products A - C were added at the rates given in Table 1. A blank feed was used as control. The diets were then pelleted at three temperatures (Table 3). Product D was diluted 1:9 with water and sprayed onto the control feed. Samples were taken before and after pelleting and assayed for enzyme activities.
The feeds were then used in an animal experiment which was carried out in two runs. A total of 576 day-old broiler chicks were divided into 12 equal groups each containing 3 male and 3 female replicates. The birds were fed the control diet for 1 week and on day 8 the groups were assigned to the experimental diets. In the first run, the feeds pelleted at 76 °C were administered, in the second set the feeds pelleted at 86 °C, and the feeds containing preparation A and control, pelleted at 72 °C. Performance parameters were recorded on day 22. Fresh digesta from birds of each treatment were taken on day 22 from the upper part of the small intestine for analysis of viscosity.
In trial 3, application processes for preparation D were investigated. The product was diluted with water of room temperature (RT) or 70 °C and sprayed in a batch process onto feed. The feeds were then transported pneumatically through an aspiration system and samples were taken before and after the transport of the feed for assay of glucanase activity.

Analytical procedures. The beta-glucanase and xylanase activities of the enzyme preparations (trial 1) were analysed by measuring liberated reducing sugars from barley glucan or carboxymethyl xylan as substrate after incubation (F. Hoffmann - La Roche, 1991). For assessing enzymatic activities in feeds (trials 2 and 3), two methods were used based on a spectrophotometric measurement of a dye liberated from a coloured substrate upon incubation of a feed extract. The concentration of the added enzyme was quantified by comparison with standard curves obtained with blank feed samples to which known amounts of enzyme had been added.
The substrate used for assessing beta-glucanase activity was a commercially available azodye-labelled barley-glucan preparation. The samples were extracted with an acetate buffer pH 4.6. The extract was incubated with the substrate at a temperature of 60 °C for 30 minutes . After precipitating the remaining insoluble substrate with a zinc acetate buffer, the solubilized dye was measured spectrophotometrically in the filtered solution at 590 nm.
The xylanase activity was measured using a commercially available dye-labelled xylan preparation. The enzyme was extracted from the feed sample using a phosphate buffer at pH 6.0. An aliquot of the extract was purified using a PD 10 sephadex G25-M column and a phosphate buffer pH 6. From the first 12 mL containing the xylanase, 2.0 mL were used for the assay with the dye-labelled substrate. The conditions for the incubation were the following: temperature 50 °C, time 2.5 h. Precipitation of the remaining substrate was achieved by the addition of a solution of hydrochloric acid in ethanol. After centrifugation, the solubilized dye was measured spectrophotometrically at 585 nm. All assays

were performed in
preparation A per l
according to Wang
contents of the feed
Statistical analysis.
animal study were
range test to deterr
Means were consid
Analytical recovery
respective supplem
enzyme preparatio
activity found in th
(Avg) and coefficie

Results and discus

The physical and ch
Table 1. Product C
all forms were suffi
homogeneous distr
thermostability, po
source of the enzyn
Table 4 gives the xy
added to the experi
xylanase and gluca
temperature, enzyn
pelleted at 76 °C, re
enzyme contents, i.
degradation caused
feeds are either fed
°C, the stability of p
The effect of varyin
preparations, anim
7. For preparation .
process temperatur
be qualitatively det
quantified. This sug
need to be elaborat
temperature, retent
preparations. This
of temperature and
In the birds given t
control in weight g
76 °C, there was no
Feed conversion wi
preparations tende
reflected in the inte
feed pelleted at 86 °
with the feed produ

were performed in duplicate. The results were expressed as mg enzyme preparation A per kg of feed. Viscosity of digesta and feed were analysed according to Wang et al., 1992. The AOAC method was used for measuring fibre contents of the feeds (Pronsky et al., 1984).

Statistical analysis. Weight, feed conversion and intestinal viscosity from the animal study were subjected to analysis of variance and Duncan's multiple range test to determine differences between means using SAS procedure. Means were considered to be significantly different at $P < 0.05$.

Analytical recovery of enzyme preparations was expressed as % of the respective supplemented plus native enzyme activities in feed. The stability of enzyme preparations in pelleted feed was expressed as retention in % of the activity found in the respective mash feed. The results are given as average (Avg) and coefficient of variation (CV).

Results and discussion

The physical and chemical characteristics of the preparations are given in Table 1. Product C was much coarser then A and B, but particle numbers/g of all forms were sufficiently high to guarantee a good mixability and homogeneous distribution of the enzymes in feeds. The forms differed in thermostability, possibly because of differing degrees of stabilisation or the source of the enzyme preparation.

Table 4 gives the xylanase and glucanase recoveries found with enzyme A added to the experimental feed. In mash feed, there was a full recovery of xylanase and glucanase activities. During storage of 1 month at room temperature, enzyme contents remained unchanged. When the feed was pelleted at 76 °C, recoveries were in the range of 75 - 90% of the expected enzyme contents, i.e. less than in the mash feed. This can be explained by a degradation caused by the pelleting process. Taken into account that most feeds are either fed as mash or as pellets not produced at temperatures over 75 °C, the stability of preparation A could be regarded as reasonably good.

The effect of varying pelleting temperatures on stability of the enzyme preparations, animal performance and intestinal viscosity is given in Tables 5 - 7. For preparation A, retention in pelleted feed decreased with increasing process temperature (Table 6). Enzyme activities of forms B and C could only be qualitatively detected and, therefore the stability of these forms could not be quantified. This suggests that with the type of assay procedure used, methods need to be elaborated for individual enzyme products. Measured at a similar temperature, retentions in pelleted feed were less than in the enzyme preparations. This suggests that enzymes are susceptible to the combined effect of temperature and moisture in the form of steam.

In the birds given the feed pelleted at 72 °C, preparation A was superior to control in weight gain and feed conversion (Table 7). With the feed pelleted at 76 °C, there was no significant effect of enzyme preparations on weight gain. Feed conversion with preparation A was superior to control, and the other preparations tended to show numerical improvements (Table 5). This is also reflected in the intestinal viscosities recorded. The enzymes A - C added to the feed pelleted at 86 °C showed generally less marked effects in the birds than with the feed produced at 76 °C. However, there was still a numerical effect of

enzymes recorded on weight gain, while form D improved feed conversion significantly (Table 7). Intestinal viscosity tended to be less with added enzymes, compared to control (Table 6).
Figure 1 summarises the findings for preparation A and D. For enzyme A, there was a parallel drop of stability and animal performance obtained with increasing pelleting temperature. While with feeds produced at 76 °C enzyme A and D performed similarly, D was superior in the feed processed at 86 °C. These results suggest that for enzyme A the critical pelleting temperature beyond which losses of activity are sizeably increased is around 75 °C. At a conditioning temperature of 75 °C, Cowan (1993) had found retentions ranging from 20% to 85%, depending on the enzyme source included in the feeds, suggesting that there are large differences of stability among commercial products.
Feed is usually not pelleted at temperatures above 75 °C. However, it has become recent practice to increase the hygienic standard of poultry feed by applying hydrothermal processes with temperatures above 80 °C. In the feed treated alike a reduced, but still present, effect of enzymes A - C was found. This is in agreement with earlier reports on improved weight gain and feed conversion recorded with expanded feed containing enzyme A (Gadient and Broz, 1992), and with long-time conditioned feed (Graham and Inborr, 1993). For feeds processed over 80 °C, the liquid enzyme D used after the hydrothermal process resulted in analytical recoveries up to the targeted addition level and tended to improve economical parameters in birds. This has also been found with glucanase enzymes, as reported by Cowan (1993). Recoveries found in the tests investigating the technical application of a liquid enzyme after the hydrothermal process are given in Table 8. When enzyme D was diluted at RT, enzyme contents of up to the target content were reached in the feeds. Uniformity of distribution ranged from 9% to 20%. On the other hand, enzyme recovery with the hot dilution was only 69%. Abrasion losses by transport of the feed containing enzyme D were up to 26%.
This indicates that liquid forms of enzymes may be used after the hydrothermal process. Suitable dosing and spraying equipment is commercially available which permits a relatively easy use of such preparations. Care must also be taken to use a precise and robust application process and to avoid losses of enzyme activity by abrasion.

Acknowledgements

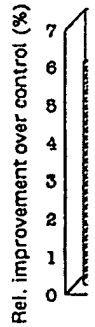
The authors wish to thank to G. Weber, CRNA Village Neuf, France, for conducting the animal study and G. Sommer for carrying out the assays of enzyme preparations.

References

Campbell, ·
teck
Nut
Cowan, W.
Inte
CRC Handl
F. Hoffmar
Gadient, M
in p
Graham, H
tion
Eur
Jongbloed,
phy
in p
Pronsky, L
B.F.
and
104
Schwarz, G
Sch
Wang, L., N
gluc
con

Fig. 1: Rel
dep

Relative i
an



References

Campbell, G.L. and Classen, H.L., 1989. Commercial application of enzyme technology in feeding barley to poultry. Proc. Pacific Northwest Anim. Nutr. Conf., Oct. 24 - 26, Boise: 66-73.

Cowan, W.D., 1993. The stability of enzymes in animal feeds. Feed International, May 1993: 22-26.

CRC Handbook of food additives, 1980. CRC Press Inc., Boca Raton Fla, USA

F. Hoffmann- La Roche, 1991. Roxazyme G, Specifications and Tests.

Gadient, M. and Broz, J., 1992. Efficacy of the enzyme complex ROXAZYME G in pelleted feed. Feed Compounder, 6: 37-38.

Graham, H. and Inborr, J., 1993. Feed enzymes: mode of action and application to heat processed poultry feeds. Proc. Amandus Kahl Seminar, Eurotier, Hannover 22.- 26.6.1993.

Jongbloed, A.W. and Kemme, P.A., 1990. Effect of pelleting mixed feeds on phytase activity and apparent absorbability of phosphorous and calcium in pigs. Anim. Feed Sci. Technol., 28: 233-242.

Pronsky, L., Asp, N.-G., Furda, I., Devries, J.W., Schweizer, T.F. and Harland, B.F., 1984. Determination of total dietary fibre in foods, food products and total diets: Interlaboratory study. J. Assoc. Off. Anal. Chem., 6: 1044-1052.

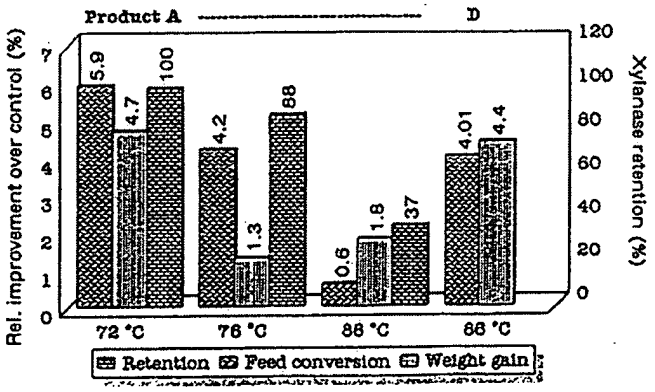
Schwarz, G. and Schöner, F.-J., 1991. Phytase in der Fütterung von Schweinen und Geflügel. Kraftfutter, 74: 163-168.

Wang, L., Newman, R.K., Newman, C.W. and Hofer, P.J., 1992. Barley b-glucans alter intestinal viscosity and reduce plasma cholesterol concentrations in chicks. J. Nutr., 122: 2292-2297.

Fig. 1: Relative improvement and stability of enzyme preparations A and D, depending on pelleting temperature of feed

---

Relative improvements and stability of enzyme preparations A and D, depending on pelleting temperature of feed



Tab. 1: Characteristics of enzyme preparations

Preparation	*A*	*B*	*C*	*D*
-Addition rate (g per kg)	0.15	1.0	1.0	0.15
-Particle size (% > 0.85 mm)	0	3	20	-
- Particle number (per g product)	3 million	1.12 million	0.13 million	-
(per 10 g of feed)	4500	11200	1300	-
- Thermostability (% retention)				
Glucanase	94%	39%	65%	-
Xylanase	98%	42%	87%	-

Tab. 2: Composition of experimental diet

Ingredient:	*%*	*Ingredient:*	*%*
Barley	30.0	Wheat	25.0
Rye	5.0	Soybean meal	27.0
Fish meal	5.0	Swine lard	4.7
DiCa phosphate	1.0	Limestone	0.9
Salt	0.2	Methionine	0.3
Vitamins	1.0		

Content (calculated):

ME (MJ/kg)	12.34	Crude protein (%):	21.9
Crude fat (%)	6.6	Crude fibre (%):	3.5
Lysine (%):	1.2	Meth. and cyst. (%):	1.0

Content (analysed, mash feed without added enzymes):

Viscosity (mPas): 0.96	Water-soluble fibres (%): 1.2
Xylanase (mg/kg): 0	Glucanase (mg/kg): 35

Tab. 3: Process conditions of experimental feeds produced at three temperatures

Pelleting temperature (°C)	*72*	*76*	*66*
Conditioning temperature (°C)	43	70	90
Throughput (kg/h)	161	161	168
Specific energy input (kWh/t)	26	22	19
Output (% maximum)	40	40	40
Moisture after pelleting (%)	11	12	14
Moisture after drying (%)	9	11	12

Tab. 4: Recover
feed (%

Feed form
Mash
Pelleted at 76 °(

Tab. 5: Perform
on enzyı

Preparation
O (Control)
A
B
C
D

a,b,c = values wi
q = qualitatively

Tab. 6: Effect of
and inte

Preparation
O (Control)
O (Control)
A
A
B
C
D

q = qualitatively

Tab. 4: Recovery of enzyme A in feed, depending on form and storage of feed (% supplemented + native enzyme activity)

Feed form	fresh				stored (30 days)	
	Xylanase		Glucanase		Glucanase	
	Avg	CV	Avg	CV	Avg	CV
Mash	103.4	16.1	107.4	5.2	107.4	8.0
Pelleted at 76 °C	91.4	29.1	76.6	8.4	75.2	14.2

Tab. 5: Performance and intestinal viscosity of broiler chicks, depending on enzyme preparation included in feeds pelleted at 76 °C

Preparation	Glucanase retention (%)	Intestinal viscosity (mPas)	Weight gain (g) day 8 - 22	Feed conversion day 8 - 22
0 (Control)	50	1.65 a	623.9 a	1.50 a
A	71	1.32 c	631.2 a	1.44 b
B	q	1.42 bc	625.4 a	1.48 ab
C	q	1.50 b	626.7 a	1.47 ab
D	120*	1.43 bc	639.6 a	1.46 ab

a,b,c = values without common letter differ significantly (P<0.05)
q = qualitatively detected ** % of supplemented + native*

Tab. 6: Effect of pelleting temperature on recovery of enzymes in feed and intestinal viscosity in broilers

Preparation	Pelleting temperature (°C)	Intestinal viscosity (mPas)	Enzyme retention Xylanase	Glucanase
			(% of content in mash feed)	
0 (Control)	72	1.60	-	100
0 (Control)	86	1.71	-	86
A	72	1.31	100	100
A	86	1.50	37	24
B	86	1.84	b	q
C	86	1.64	b	q
D	86	1.39	q	116*

q = qualitatively detected *b = below detection limit* ** % supplemented + native*

Tab. 7: Performance of broiler chicks, depending on enzyme preparation included in feeds pelleted at various temperatures

Preparation	Pelleting temperature (°C)	Weight gain (g) day 8 - 22	Feed conversion day 8 - 22
0 (Control)	72	586.6 a	1.61 a
0 (Control)	86	591.7 a	1.57 a
A	72	614.4 a	1.52 b
A	86	602.7 a	1.56 a
B	86	603.2 a	1.57 a
C	86	607.0 a	1.56 a
D	86	618.0 a	1.51 b

a,b = values without common letter differ significantly (P<0.05)

Tab. 8: Recovery of liquid enzyme preparation D in broiler feeds, depending on application process

Temperature (°C) Pellets/Dilution	Recovery in feed (% of supplemented + native enzyme activity)		Recovery after transport (% of content before transport)
	Avg	CV	Avg
82/20	113.3%	9.2%	97.6%
82/70	68.7%	19.7%	74.4%

THERMOSTABILITY
PROCESSES AND APP

Dr. William D. Co
Technical Manager
Novo Nordisk Bio-
and
Dr. Peter B. Rasmu
Laboratory Manage
Novo Nordisk A/S,

SUMMARY

An analysis metho
the levels of enz
pre and post pell
(Analysis, Enzymes

INTRODUCTION

Recent studies ha
to improve the n
(Annison 1992, C
studies with chic
experience with e
the reported inve
used mash or low
discrepancy in
processing.

As feed enzymes
above 65°C it is
of enzymes can t
stabilisation tha
substrate. Howev
sensitive enough

The present inve
which could dete
after pelleting.
thermal stability
method should be
different sources

ASSAY METHODS

Four alternative
standard assay me
xylan substrate)
liberated reducin
and extraction
extended incubati

Comparison of th
addition rates of
method was not
detection limit.
of addition but
readily available
enzymes. Measurem